D-002: Effects on Hepatic Drug Metabolizing Enzyme Activities in Rats

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ABSTRACT

D-002 is a new natural drug from beeswax with anti-inflammatory and anti-ulcer effects proved in different experimental animal models. Pre-clinical toxicology of D-002 showed no drug-related toxicity. The present study was performed to evaluate the effects of D-002 on drug-metabolizing enzymes (Fase I) in male Sprague Dawley rats. The content of P-450 and b_5 cytochromes and the activities of the NADPH cytochrome c reductase, aminopyrine *N*-demethylase, *N*-dimethylnitrosamine dealkylase, were determined. The biotransformation of benzo(a)pyrene was evaluated by using the Ames test. The results demonstrate that D-002 administered orally at dosis of 50 to 1000 mg/kg/day for one month does not affect the activities of the hepatic drug-metabolizing enzymes investigated. Positive controls treated with phenobarbital and β-naphthoflavone showed significant differences.

Keywords: D-002, hepatic drug-metabolizing enzymes, Sprague Dawley rats

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RESUMEN

D-002: efectos sobre la actividad de las enzimas hepáticas metabolizadoras de medicamentos en ratas. En este trabajo se evaluaron los posibles efectos de D-002, un nuevo producto natural aislado y purificado de la cera de las abejas que presenta actividad antiinflamatoria y antiulcerosa en diferentes modelos animales experimentales. El estudio toxicológico preclínico no mostró toxicidad relacionada con el medicamento. El presente trabajo se realizó con el objetivo de estudiar los posibles efectos del producto sobre algunas enzimas microsomales hepáticas de fase I en ratas macho Sprague Dawley. Se evaluaron los efectos sobre el contenido total de citocromo P-450, citocromo b_5 y sobre las actividades de las enzimas citocromo c-reductasa dependiente de NADPH, aminopirina-desmetilasa y *N*-dimetilnitrosamina-desalquilasa. Además, se evaluaron los efectos del producto sobre la biotransformación del benzo(a)pireno mediante la prueba de Ames. Los resultados demuestran que la administración de dosis orales de D-002 (50-1 000 mg/kg/día) durante 30 días no modifica el contenido de los citocromos P-450 y b_5, ni la actividad enzimática de los sistemas estudiados. Sin embargo, las fracciones microsomales procedentes de los animales del grupo utilizado como control positivo que fue tratado con fenobarbital y β-benzoflavona, sí mostraron los cambios esperados en todos los casos.

Palabras claves: D-002, enzimas hepáticas metabolizadoras de medicamentos, ratas Sprague Dawley

*I*ntroduction

D-002 is a mixture of primary aliphatic alcohols of high molecular weight isolated and purified from beeswax *(Apis mellifera)*. It contains triacontanol followed by octacosanol, dotriacontanol, hexacosanol and tetracontanol, and tetratriacontanol as a minor component. D-002 has shown mild anti-inflammatory activity [1] and effective anti-ulcer effects in different experimental animal models [2, 3]. D-002 administered orally at 5 to 50 mg/kg prevented ulcers experimentally induced by ethanol (60%), HCl (0.6 M) and indometacin. It also inhibits ulcers induced in pylorus-ligated rats [2]. These anti-ulcer effects seem to be mediated by a reinforcement of the gastric mucosa defensive mechanisms [3].

Acute, subchronic and chronic toxicity studies in rats did not show any drug-related toxicity [4, 5]. Likewise, *in vitro* and *in vivo* mutagenicity studies did not show genotoxic effects on somatic or germinal cells [6]. Some anti-ulcer drugs like H_2 antagonists and the Na^+/K^+ pump inhibitors act as inductors or inhibitors of different families of the hepatic mixed-function oxidase system [7–9], which implies that the study of drug-metabolizing enzyme activities of new drugs with anti-ulcer effects is still justified.

The aim of this research was to study whether D-002 administered orally at 50–1000 mg/kg/day for one month affects the activity of this oxidase system in male Sprague Dawley rats.

*M*aterials and Methods

Chemicals

All chemicals were of analytical reagent grade. Phenobarbital, NADP and NADH (sodium salts) were obtained from BDH (UK). Acacia gum, β-naphthoflavone, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cytochrome c, NADPH, aminopyrine, dimethylnitrosamine, benzo(a)pyrene and sodium dithionite were purchased from Sigma (St. Louis, MO, USA).

Animals

Male Sprague Dawley rats weighing 150 to 200 g were obtained from the Centro Nacional para la Producción de Animales de Laboratorio (CENPALAB, Havana, Cuba). Animals were adapted to experimental conditions.

The water and food (rat standard chow, supplied by CENPALAB) were supplied *ad libitum.*

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Administration, dosage and experimental groups

Relative concentrations of each alcohol in the D-002 batch were: triacontanol (26.63%), octacosanol (17.49%), dotriacontanol (16.95%), hexacosanol (15.39%), tetracosanol (13.24%) and tetratriacontanol (2.23%). All the suspensions were prepared daily in acacia gum-water vehicle (10 mg/kg). The drugs were administered daily for a month by oral gavage.

Three experimental groups of 12 animals each were treated with 50, 500 and 1 000 mg/kg/day of D-002. Control rats received by gavage similar volumes of the vehicle used in the preparation of the suspensions. Other two animal groups were administered in the study: one was used as positive control and was administered with phenobarbital and β-napthoflavone according to INVITTOX Protocol [10]; the other was used as negative control and received no treatment.

Twenty-four hours after the last dose, each rat was weighed, sacrificed by cervical dislocation, bled and placed on its back on an autopsy board. Livers were removed, weighed and used for microsome preparation. The weight of the liver was used to obtain the percent of organ weight relative to body weight (%). It was calculated as follows:

% = weight of liver/body weight x 100

Preparation of microsomes

Liver samples (5 g) from three animals were homogenized in three volumes of ice-cold 1.15% KCl using a Polytron homogenizer. The cell debris, nuclei and mitochondria were removed by centrifugation at 9 000 x*g* for 20 min at 0–4 ºC. The supernatant was ultracentrifuged at 100 000 x*g* for 60 min at 0–4 ºC. The pellet was resuspended in ice-cold 0.1 mol/L Tris-HCl buffer, pH 7.4 containing 0.175 mmol/L KCL and 0.2 mmol/L EDTA. Aliquots of 1 mL of microsomal fractions were quickly stored at -80 ºC until usage. Sterility of the preparation was determined by plating 0.1 mL on minimal agar containing histidine and biotin.

Microsomal proteins were determined according to the method of Lowry *et al.* [11]. P-450 and b_5 cytochromes were determined by the method of Omura and Sato [12], and NADPH cytochrome c reductase activity as described by Williams and Kamin [13]. Aminopyrine demethylase activity was measured through formaldehyde formation according to the method of Nash [14] and dimethylnitrosamine *N*-demethylase according to Weibel *et al.* [15].

Ames mutagenicity test was used as indicator of the benzo(a)pyrenohydroxilase activity. The bacteria *(Salmonella typhimurium*, TA38 strain), the benzo(a)pyrene (10 and 20 µL/plate), the cofactors and the activation system (containing 20% v/v liver microsomes) were incubated at 37 ºC for 48 h [16].

Statistical analysis

Data are presented as mean values ± standard deviations of the mean. Comparisons between groups were performed using the Mann-Whitney test, with $\alpha = 0.05$ selected a priori for statistical significance.

Results and Discussion

In this study, the effects of a new mixture of higher primary aliphatic alcohols on the microsomal mixed-funtion oxidase system in rats were investigated. The effects of repeated oral doses of D-002 during a month on the parameters measured are shown in Tables 1 and 2. As it can be observed, D-002 did not affect liver weight, which is in agreement with the results of previous toxicological studies conducted in Sprague Dawley rats [5]. No differences were observed between treated and control groups relating to the rest of the parameters measured. These results show that D-002 did not affect hepatic microsomal drug-metabolizing enzyme activity even at 1 000 mg/kg/day.

The total content of the cytochromes P-450 and b_5 was not modified indicating that D-002 did not act through a direct interaction with them. No significant difference in the NADPH-dependent reduction of cytochrome c was detected, which indicates that D-002 did not interfere with the electron flow to the cytochromes. Likewise, the forms of the cytochromes

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Table 1. Effects of oral administration of D-002 during a month to male Sprague Dawley rats on hepatic microsomal drug metabolizing enzyme activity.

Group	Liver weight (%)	Protein (mg/kg)	Cytochrome P-450 (nmol/mg)	Cytochrome b_5 (nmol/mg)
Control (-)	2.52 ± 0.28	25.6 ± 0.8	0.41 ± 0.05	0.43 ± 0.07
Control (+)	3.82 ± 0.24*	31.2 ± 2.1*	1.76 ± 0.15*	0.73 ± 0.08*
Control vehicle	2.52 ± 0.15	26.7 ± 2.9	0.47 ± 0.13	0.50 ± 0.10
D-002				
50 mg/kg	2.62 ± 0.20	26.8 ± 0.8	0.47 ± 0.10	0.52 ± 0.11
500 mg/kg	2.60 ± 0.21	28.6 ± 2.1	0.51 ± 0.10	0.52 ± 0.09
1000 mg/kg	2.69 ± 0.29	29.0 ± 2.1	0.46 ± 0.05	0.49 ± 0.10

	NADPH cyt c reductase (nmol/mg/min)	Aminopyrine demethylase (nmol/g liver)	Dimethylnitrosamine dealkylase (nmol/mg/min)
Control (-)	15.6 ± 2.8	0.12 ± 0.03	0.08 ± 0.01
Control (+)	26.7 ± 9.4*	0.46 ± 0.10*	0.15 ± 0.03*
Control vehicle	14.5 ± 2.1	0.11 ± 0.05	0.09 ± 0.01
D-002			
50 mg/kg	14.8 ± 3.1	0.09 ± 0.02	0.08 ± 0.02
500 mg/kg	15.7 ± 1.6	0.12 ± 0.02	0.08 ± 0.01
1000 mg/kg	14.6 ± 3.6	0.14 ± 0.03	0.09 ± 0.03

Control (-): microsomal fraction obtained from non-treated animals. Control (+): microsomal fraction obtained from animals treated with phenobarbital and β-napthoflavone. %: percent of the liver weight relative to body weight. Results as mean ± SD, Mann Whitney U test, *p < 0.05.

Table 2. Results of oral administration of D-002 during a month to male Sprague Dawley rats on the benzo(a)pyrene biotransformation using the Ames test.

	Concentration of benzo(a)pyrene	
	10 µL/plate	20 µL/plate
S_9 mix from non-treated animals	25.3 ± 2.5	24.6 ± 0.6
S_9 mix from induced animals	47.0 ± 6.2*	77.3 ± 2.1*
S_9 mix from vehicle treated animals	22.0 ± 1.7	24.6 ± 1.5
S_9 mix from 50 mg/kg treated animals	22.6 ± 1.7	23.7 ± 0.6
S_9 mix from 500 mg/kg treated animals	20.3 ± 6.5	22.3 ± 0.6
S_9 mix from 1000 mg/kg treated animals	22.6 ± 2.3	24.3 ± 1.5

The data represent the number of revertant colonies/plate of almost three independent experiments (3 plates/dose), (x ± SD). The strain of *Salmonella typhimurium* used was TA38, S_9 mix contained 20% v/v liver microsomes and cofactors. Mann Whitney U test, *p < 0.05.

P-450 responsible for the biotransformation of the substrates used were not increased nor decreased. In this case, we determined the activity of CYP3A, CYP2E and CYP1A enzymes through the biotransformation of benzo(a)pyrene using the Ames test. These represent some of the main isoenzymes of this superfamily of proteins with toxicological or clinical implications [17].

By contrast, the expected increase in these parameters in rats treated with phenobarbital and β-benzoflavona was observed. These findings support the validity of the methodologies and conditions used in this work.

Few studies have been performed to gain insight into the biotransformation of these very long chain aliphatic alcohols. Nevertheless, results of pharmacokinetic and metabolic studies have suggested that they may be partly oxidized and degraded to fatty acids through β-oxidation, although no strong evidences are available [18, 19]. Previous studies performed with policosanol (another mixture of very long chain aliphatic alcohols) demonstrated that treatment with this drug does not interfere with the activity of the drug-metabolizing enzymes in rats nor in dogs [20, 21]. These premises and the results of this work suggest that the metabolism of this kind of chemicals does not induce changes on the hepatic microsomal system.

On the other hand, H_2 antagonists and Na^+/K^+ pump inhibitors affect the content of cytochrome P-450 and the several enzymatic activities of these monooxygenase systems in rat liver microsomes [7, 9]. However, D-002 did not affect hepatic microsomal drug-metabolizing enzyme activities, which is not surprising because the anti-ulcer action mechanism of D-002 differs from that of these drugs.

The microsomal cytochrome P-450 dependent mixed-function oxidase system comprises a superfamily of protein with one or more isoenzymes each [22, 23]. Many chemicals may lead to selective increase or decrease of the activities of one or more families. This is very important in pre-clinical assays of new drugs because it could be relevant for clinical use [24]. Drug interactions during therapy may lead to significant toxicity or treatment failure and one of the primary mechanisms for the development of interactions is the perturbation of one or multiple hepatic enzymes that make up the cytochrome P-450 detoxifying system. In addition, the information on whether the drug can adversely affect an enzyme pathway will also help to anticipate potential drug interactions.

The results of this study indicate that D-002 does not induce any changes on the hepatic drug-metabolizing activities measured.

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